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Distribution of Dorsal-Forming Activity in Precleavage Embryos of the Japanese Newt, *Cynops pyrrhogaster*: Effects of Deletion of Vegetal Cytoplasm, UV Irradiation, and Lithium Treatment

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Two types of axis-deficient embryos developed after deletion of the vegetal cytoplasm: wasp-shaped embryos and permanent-blastula-type embryos. *In situ* hybridization revealed that neither type of axis-deficient embryo expressed *goosecoid* or *pax-6. brachyury* was expressed in the constricted waist region of the wasp-shaped embryos but was not expressed in the permanent-blastula-type embryos. Further, we examined the effect of UV irradiation on Japanese newt embryos. Surprisingly, UV-irradiated Japanese newt eggs formed hyperdorsalized embryos. These embryos gastrulated in an irregular circular fashion with *goosecoid* expression in the circular equatorial region. At tailbud stage, these embryos formed a proboscis which is very reminiscent of that formed in hyperdorsalized *Xenopus* embryos. Transplantation of the marginal region of the UV-irradiated embryos revealed that the entire marginal zone had organizer activity. Thus we conclude that UV hyperdorsalizes Japanese newt embryos. Finally, lithium treatment of normal embryos at the 32-cell stage also resulted in hyperdorsalization. Lithium treatment of vegetally deleted embryos had two distinct results. Lithium treatment of permanent-blastula-type embryos did not result in the formation of dorsal axial structures, while the same treatment reinduced gastrulation and dorsal axis formation in the wasp-shaped embryos. Based on these results, we propose a model for early axis specification in Japanese newt embryos. The model presented here is fundamentally identical to the *Xenopus* model, with some important modifications. The vegetally located determinants required for dorsal development (dorsal determinants, DDs) are distributed over a wider region at fertilization in Japanese newt embryos than in *Xenopus* embryos. The marginal region of the Japanese newt embryo at the beginning of development overlaps with the field of the DDs. Gastrulation is very likely to be a dorsal marginal-specific property, while self-constriction is most probably a ventral marginal-specific property in Japanese newt embryos.

INTRODUCTION

Formation of the dorsal–ventral axis of the vertebrate embryo is one of the most interesting events in animal development. In amphibians, dorsal axis formation is thought to be the result of early developmental events, the most famous of which is the organizer phenomenon explored originally by Spemann and Mangold (1924). Significant advances have recently been made in understanding the early steps in formation of the *Xenopus* organizer.

In *Xenopus* development, the first event important for dorsal axis formation is rotation of the cortical cytoplasm, which accurately specifies the direction of the dorsal–ventral axis of the embryo (Gerhart *et al.*, 1989; Vincent and Gerhart, 1987a,b; Vincent *et al.*, 1986). Deletion of the vegetal but not the lateral cytoplasm of *Xenopus* eggs early in the first cell cycle results in a complete loss of the dorsal

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axis (Kikkawa *et al.*, 1996; Sakai, 1996). This axis deficiency can be completely rescued by the injection of an amount of vegetal cytoplasm (Sakai, 1996). However, when the vegetal deletion is instead carried out late in the first cell cycle (just before the first cleavage), the resulting embryos usually form a normal dorsal axis (Sakai, 1996). Injection of cortical cytoplasm from the vegetal pole region into the ventral subequatorial region of recipient embryos results in the formation of a secondary dorsal axis (Fujisue *et al.*, 1993; Holowacz and Elinson, 1993, 1995). Further, this dorsalizing activity is shown to be present in the cell cortex (Kageura, 1997). After the first cleavage, this activity disappears from the vegetal region and reappears in the dorsal region, without any dorsal-inducing activity being detected in the ventral subequatorial region at any stage (Fujisue *et al.*, 1993; Yuge *et al.*, 1990). Thus, the dorsal determinants are sufficient (Fujisue *et al.*, 1993; Holowacz and Elinson, 1993, 1995; Yuge *et al.*, 1990) and necessary (Sakai, 1996) for axis formation in the marginal zone. It has been proposed that the vegetally localized dorsal determinants are translocated, by cortical rotation, to the equatorial region, leading to formation of the organizer (Sakai, 1996). UV irradiation ventralizes *Xenopus* embryos, probably by inhibiting cortical rotation (Elinson and Kao, 1989; Elinson and Pasceri, 1989; Scharf and Gerhart, 1983; Scharf *et al.*, 1989). In these UV-irradiated embryos, the dorsal determinant does not move to the equatorial region (Fujisue *et al.*, 1993), which most probably causes the inhibition of Spemann organizer formation (Sakai, 1996). Lithium treatment, which may bypass the dorsal-determinant pathway, hyperdorsalizes both normal (Kao and Elinson, 1988, 1989; Kao *et al.*, 1986) and vegetally deleted (Sakai, 1996) *Xenopus* embryos.

The axis formation processes undergone by *Xenopus* and Japanese newt embryos have certain features in common. As in *Xenopus*, cortical rotation accurately specifies the dorsal–ventral axis in Japanese newt embryos (Fujisue *et al.*, 1991), and in both species the region around the dorsal gastrulating cells, i.e., the Spemann organizer cells, specifically induces a secondary axis when transplanted into the ventral region. However, the two species show different gastrulation patterns. In *Xenopus*, the entire marginal zone forms gastrulating cells (bottle cells), and ventralized *Xenopus* embryos retain the ability to form pigmented bottle cells (Sakai, 1996; Scharf and Gerhart, 1980). In contrast, the ventral marginal zone of the Japanese newt embryo does not show pigment concentration even at the end of gastrulation. This is most likely a specific characteristic of the Japanese newt, *Cynops pyrrhogaster*. Thus there appear to be differences in the gastrulating activity in the ventral region of the two species. The determinant hypothesis for *Xenopus* described above (Sakai, 1996), therefore, may or may not be directly applicable to Japanese newt embryos, so we decided to examine the effect of vegetal deletion, UV irradiation, and lithium treatment on Japanese newt embryos, all of which have previously been studied in *Xenopus* embryos in recent years.

Based on our results, and by comparison with current models of *Xenopus* development, a model of the early axis formation process in Japanese newt embryos is proposed.

MATERIALS AND METHODS

Preparation of Eggs and Sperm

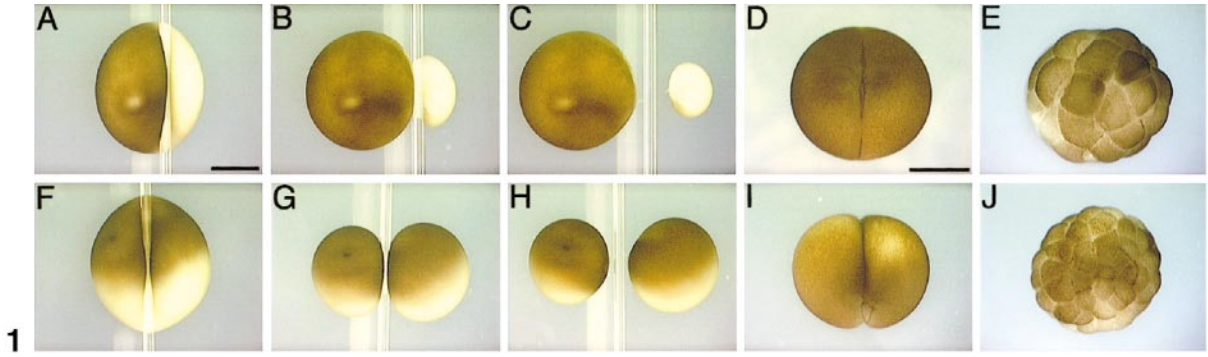
Adult Japanese newts, *C. pyrrhogaster*, were purchased from Hamamatsu seibutsu kyouzai (Hamamatsu, Japan) or collected in the suburbs of Kagoshima city. The animals were kept at room temperature and given beef liver twice a week or were stored at 5°C without feeding until use.

Ovulation was induced in females by several daily injections of human chorionic gonadotropin (Teikoku Zoki Co.; 50 iu per animal). Unfertilized eggs were squeezed from spawning females. Seminal fluid was squeezed from males with violet tails (nuptial coloration) and diluted, if necessary, with 10% modified Steinberg's solution (MS; 100% MS consists of 58.2 mM NaCl, 0.67 mM KCl, 0.34 mM $Ca(NO_3)_2$, 0.83 mM $MgSO_4$, and 3.0 mM Hepes–NaOH, pH 7.4). Staging of the embryos was carried out according to Okada and Ichikawa (1947). Insemination was performed by the method of Fujisue *et al.* (1991). Eggs were placed in a dry petri dish (5.4 cm diameter), and each was fertilized by the application of a small drop of sperm on the equator of the egg, using fine forceps under a dissecting microscope. Seven to ten minutes later, 10 ml of 10% MS was added to the eggs.

Deletion of the Vegetal Portion of the Egg

Fertilized newt eggs were dejellied 30 min after insemination using 1% sodium thioglycolate in 10% MS, pH 9–10, rinsed with 10% MS, and demembranated manually 2–3 h after insemination. The denuded egg was inclined at 90° to its vertical axis and an agar-coated glass rod (diameter 200–250 μm, length 25 mm) was placed onto the egg to divide it into one animal and one vegetal fragment (vegetal deletion; Figs. 1A–1E) or to divide it into a sperm entrance point (SEP) side and an anti-SEP side fragment (vertical deletion; Figs. 1F–1J). Usually, the SEPs could be seen on the sperm-applied side of the embryo at the time of placement of the glass rod (the small black spot in Figs. 1F–1H). The weight of the glass rod exerted sufficient pressure to gradually constrict the egg, causing it to separate into two fragments with no cytoplasmic debris (Fig. 1). Both fragments maintained a spindle shape after complete separation (Figs. 1C and 1H). The lengths of their long and short axes were measured using a micrometer attached to the eyepiece of a microscope while the surface area of the fragments was calculated by assuming that they were spheroids. The degree of deletion was evaluated as the ratio of the surface area of the deleted fragment relative to the sum of the surface areas of both fragments. After complete separation, nucleus-containing animal or SEP side fragments were placed in round wells in a petri dish coated with 2% agar.

Deletions were carried out at time 0.2 to 0.4 on a normalized time scale, in which fertilization is defined as time 0 and the time of first cleavage (8–9 h after fertilization) is given the arbitrary value 1.0. At times later than 0.5, the egg gradually gained stiffness so that the previously used glass rod was not enough to constrict the egg, while a heavier glass rod caused instant rupture of the egg membrane. Therefore we could not examine the effect of deletion at later stages.



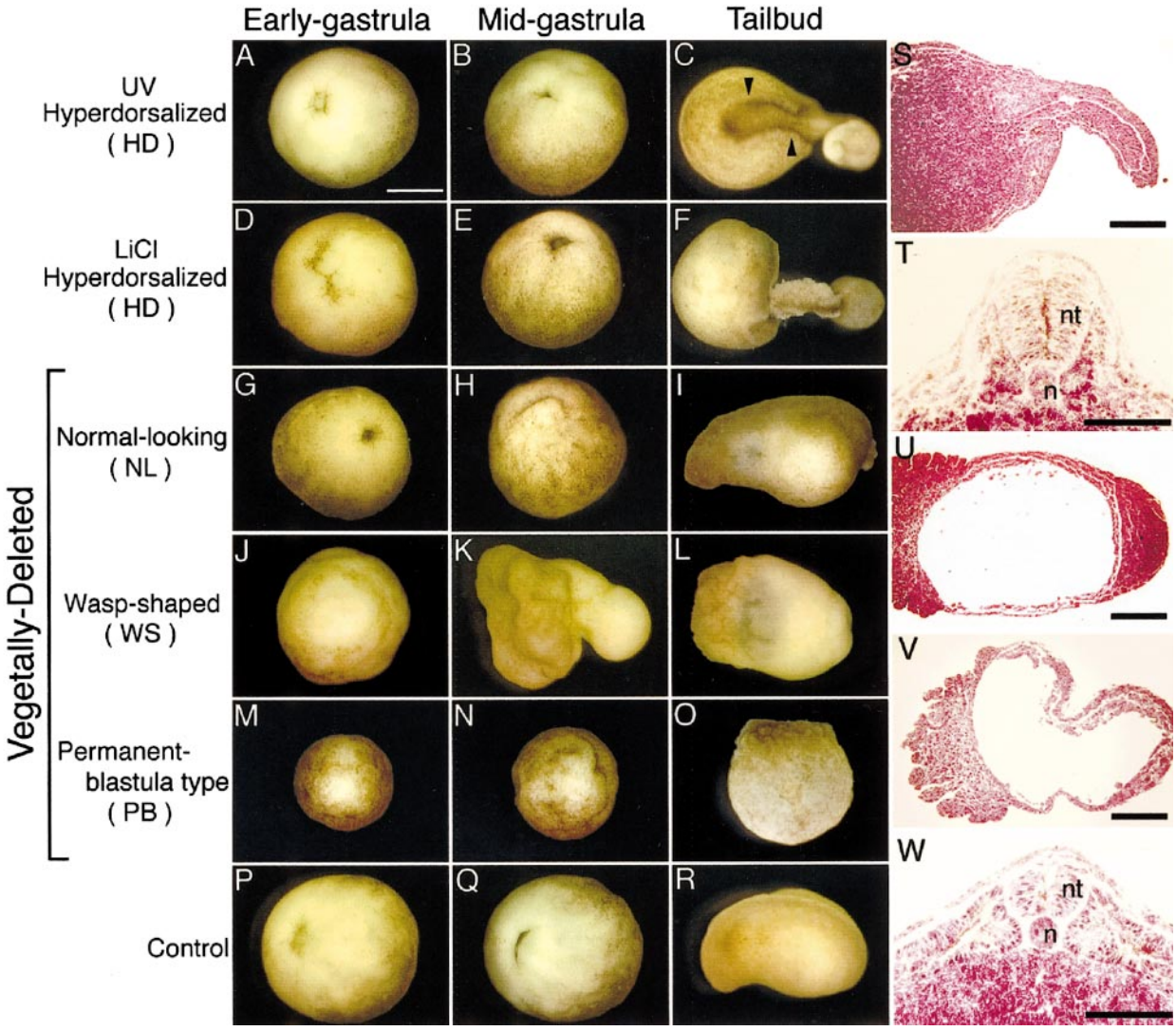


FIG. 1. Deletion of vegetal or lateral cytoplasm from newt embryos. (A–E) Vegetal deletion. In the experiment shown, a glass rod was placed on the egg at time 0.29 (153 min after fertilization), and 19% of the egg surface area was deleted. (F–J) Lateral deletion. In this instance, deletion was started at time 0.31 and 57% of the egg surface was deleted. (A, F) Just after placing the glass rod in position. (B, G) After 15–30 min, the glass rod constricts the eggs so that they take on a dumbbell shape. (C, H) Just after completion of the separation. (D, I) First cleavage. (E, J) At 32–64 cells. Note that both fragments cleaved normally. Bar in A for A–C and F–H, 1 mm. Bar in D for D, E, I, and J, 1 mm.

FIG. 3. External morphology of the deleted embryos. The abbreviations for the class of morphology and deleted volumes are shown in parentheses. For example, (NL, 11) denotes that the embryo was scored as NL and the deleted volume was 11% of the total surface area. (A) Tailbud stage embryos (stage 25) resulted from vegetal deletion. Embryos developed into NL, -0, and -NG embryos depending on the deleted volume (see Fig. 4 for precise data). (B) Vertical deletion. All embryos developed into NL embryos. Cont., a control embryo. (C) Vegetally deleted WS embryos at stage 12b. (D) The same embryos as in (C) at stage 13, when constriction of the waist (arrowheads) reached a maximum. All bars, 1 mm.

UV Irradiation

Irradiation was carried out using a Mineralite UVGL-25 source (Ultraviolet Products, San Gabriel, CA) emitting predominantly at 2537 Å.

Dejellied, fertilized eggs were placed on a quartz slide at a time of approximately 0.4 (3 h after fertilization) and positioned uniformly 2 cm from the irradiation source. The eggs naturally oriented themselves with their vegetal hemisphere facing downward. Irradiation was generally for 75 s, at which time the eggs' vegetal poles had received 1.2×10^4 ergs/mm^2. Cortical rotation of the irradiated embryos was examined together with nonirradiated controls (data not shown).

Lithium Treatment

At the 32-cell stage, early vegetally deleted and control embryos were treated with 0.3 M LiCl for 20 min. They were then washed

FIG. 2. Morphology of hyperdorsalized (HD), normal-looking (NL), wasp-shaped (WS), and permanent-blastula-type (PB) embryos. The classifications were made at the tailbud stage (stage 25). (A–C) An HD embryo which resulted from UV irradiation. (D–F) An HD embryo resulting from LiCl treatment. (G–I) An NL embryo. This is a vegetally deleted embryo, from which a relatively small volume (19% of the surface area in this case) was deleted. (J–L) WS embryo obtained by vegetal deletion (24% in this case). (M–O) PB embryo obtained by vegetal deletion (72% in this case). (P–R) Normal control embryos. (A, D, G, J, M, P) At the onset of gastrulation (stage 11). Vegetal view. (B, E, H, K, N, Q) Ten to twelve hours after the onset of gastrulation (stage 12b). Vegetal view except for K (side view). (C, F, I, L, O, R) At the tailbud stage (stage 25). Side view. (S–W) Histological sections of HD (UV-irradiated), NL, WS, PB, and control embryos at the tailbud stage. Arrowheads in C, neural-fold-like structure. Bar in A for A–R, 1 mm. Bars in S–W represent 0.5 mm.

thoroughly with 10% MS and allowed to develop in an agar well until the controls reached tailbud stage.

Morphological Analysis: Four Classes of Axis Development in Japanese Newt Embryos

The external morphology of all the experimental embryos was examined when control embryos reached the tailbud stage. In addition, some embryos were fixed for histological examination.

In order to assess the dorsoanterior development of *Xenopus laevis*, Kao and Elinson (1989) defined a scale termed the dorsoanterior index (DAI), in which axial variation is classified into 11 categories (DAI 0 to 10). In our newt embryos, axial variation was difficult to classify into such specific DAI grades. Hyperdorsalized and ventralized newt embryos are very sensitive and fragile, so that it was very difficult to rear them until the tadpole stage at which time axis formation can be determined by the formation of eyes as in *Xenopus* embryos. However, despite this sensitivity, some of the experimental embryos were successfully reared until tadpole stage. Our results showed that once the Japanese newt embryos formed a dorsal axis, they developed into fairly normal embryos with at least a fused eye. We therefore examined embryo morphology at the tailbud stage and divided the experimental embryos into the following four classes: hyperdorsalized (HD) embryos, normal-looking (NL) embryos, wasp-shaped ventralized (WS) embryos, and permanent-blastula-type (PB) embryos (Fig. 2).

To examine internal morphology, several experimental embryos were fixed in Bouin's fixative, dehydrated through an ethanol series, and embedded in Paraplast. They were serially sectioned at 8 μm and stained with hematoxylin and eosin.

Whole-mount in Situ Hybridization

Whole-mount *in situ* hybridization (Harland, 1991) was performed with the modifications described by Sone *et al.* (1997). Hybridization was carried out using the *goosecoid* and *brachyury* probes described by Sone *et al.* (1997) and the *pax-6* probe described by Mizuno *et al.* (1997a). Embryos were fixed in MEMFA overnight at 4°C, washed, and stored in 100% methanol. After satisfactory color development, embryos were treated overnight in 10% hydrogen peroxide to bleach out the pigment.

RESULTS

Deletion of Vegetal but Not Lateral Cytoplasm Early in the First Cell Cycle Results in Two Types of Axis-Deficient Embryo

Deletion of cytoplasm from both the vegetal and the lateral regions using the method described previously in this paper could be achieved without any additional loss of cytoplasm from the remaining embryo at times between 0.2 and 0.4. Most of the resulting embryos cleaved at a rate identical to their normal counterparts (Figs. 1E and 1J). Cortical rotation was observed in vegetally deleted newt embryos (n = 14, data not shown) although the extent of the movement was small.

Vegetal deletion resulted in two types of axis-deficient embryo: wasp-shaped ventralized embryos and permanent-blastula-type embryos (Figs. 2 and 3). When 25–40% of the surface area was deleted (Fig. 4), most of the resulting embryos did not gastrulate but underwent a self-constriction movement which led to wasp-shaped embryos (Figs. 3A, 3C, and 3D). To our knowledge, this form has not been reported in any amphibian embryo to date. We defined these embryos as WS. At the onset of gastrulation in the normal control embryos, WS embryos did not show any sign of morphological change (Fig. 2J); however, they exhibited characteristic morphogenesis during the normal gastrulation period. Three to five hours after the start of gastrulation in the controls, the vegetal region of the WS embryos began to change shape. The vegetal pole extruded and the pigment boundary (boundary of the animal black area and the vegetal white area) was constricted so that the embryo took on a narrow-waisted wasp-like shape (Figs. 2K and 3C). The constriction gradually regressed after reaching a maximum at stage 13 (Fig. 3D), so that by the tailbud stage the embryo had an exogastrula-like appearance (Figs. 2L and 3A). Histological sections at this stage showed that these embryos consisted of yolky endodermal (Fig. 2U, right side) and pigment-containing epidermal cells (Fig. 2U, left side). No neural or mesodermal cells were found (Fig. 2U). These embryos do not resemble any type of dorsally enhanced or dorsally reduced *Xenopus* embryo. We suspect that these embryos are analogous to DAI-0 (ventralized) *Xenopus* embryos for several reasons, which are described later in this paper.

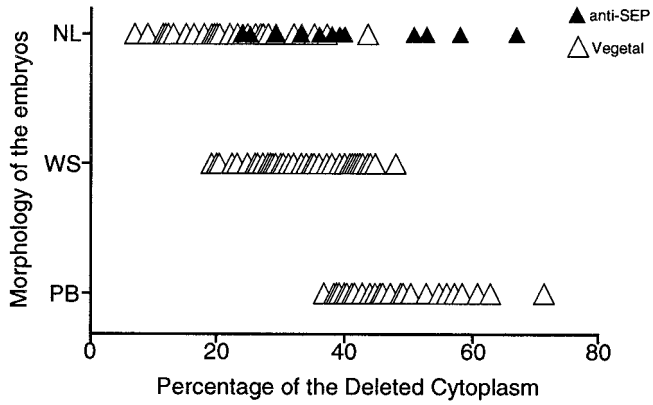


FIG. 4. The effect of early vegetal and vertical (anti-SEP) deletions. Data from individual embryos are plotted against the deleted area (% of the whole surface). Data from embryos undergoing deletion before time 0.2–0.4 (start of deletion) are shown. Embryos that completed separation after the first cleavage (time 1.0) were omitted. Open triangles, vegetal deletion. Black triangles, vertical (anti-SEP) deletion.

The other type of axis-deficient embryo observed in our study was the PB embryo, which formed when more than 35% of the egg surface was deleted (Fig. 4). These embryos neither formed blastopores (Fig. 2M) nor underwent self-constriction movement (Fig. 2N). PB embryos developed into "permanent-blastula" embryos which consisted en-

tirely of epidermal cells (Figs. 2O and 2V). PB embryos most likely have no counterpart in the *Xenopus* DAI categories, but are probably comparable to the *Xenopus* NG (nongastrulating) embryos, which we have described previously (Sakai, 1996).

When the deleted vegetal portion was relatively small (0 to 20%), the resulting embryos usually gastrulated normally (Figs. 2G and 2H) and developed into NL embryos (Figs. 2I and 4). When carefully reared, these embryos developed into tadpoles with two normal eyes or a single fused eye. These embryos are likely to be analogous to DAI-3, -4, and -5 (normal) *Xenopus* embryos.

The loss of dorsal structures as a result of deletion of cytoplasm was specific for only vegetal deletions, since lateral (vertical) deletions did not cause any dorsal deficiencies (Fig. 3B), as is also the case with *Xenopus* deletions (Sakai, 1996). The laterally deleted embryos developed normally until the tailbud stage, an observation previously reported by Kobayakawa and Kubota (1981). Of 14 laterally deleted embryos in which 25 to 67% of the egg surface was deleted, all 14 developed into NL embryos ("anti-SEP" in Fig. 4).

As shown in Figs. 3A and 4, the degree of loss of dorsal structures was dependent on the volume of vegetal cytoplasm deleted. Compared to *Xenopus* embryos, a greater volume must be deleted from newt embryos for complete axis deficiency (WS and PB). Some newt embryos formed dorsal structures even when 43% of the vegetal cytoplasm was deleted (Fig. 4). As seen in Fig. 4, the ranges of vegetal cytoplasm deletions resulting in PB, WS, and NL embryos had a very large overlap compared to the ranges of *Xenopus*. In *Xenopus*, a deletion of 15–40% inevitably resulted in DAI-0 embryos (Sakai, 1996). However, with the Japanese newt, a deletion of 19–37% resulted in embryos that developed into either WS or NL embryos, while in the 43–48% range, the resulting embryos developed into WS or PB embryos. All three types of embryo developed when 37–48% of the cytoplasm was deleted.

In Situ Hybridization of Vegetally Deleted Embryos

We next analyzed the deleted embryos by whole-mount *in situ* hybridization using *goosecoid* (dorsal marginal marker), *brachyury* (general mesodermal marker), and *pax-6* (neural marker) probes. Since the normal expression of *goosecoid* is best observed at stage 12b and *brachyury* at stage 13 (Sone *et al.*, 1997), experimental embryos were fixed at these two stages. At stages 12b and 13, NL, WS, and PB embryos could be distinguished by external morphology: NL embryos formed a blastopore, WS embryos showed self-constricting movement, and PB embryos did not show either characteristic (Fig. 2). Some embryos were fixed at stage 25 (tailbud stage) in order to examine the expression of *pax-6*, which is normally expressed in the eye and along the discrete regions of the neural tube at this stage (Mizuno *et al.*, 1997a).

As shown in Fig. 5, WS and PB embryos showed no *goosecoid* expression, and *pax-6* was not observed in either type of embryo at the tailbud stage. These results confirm that WS and PB embryos do not form dorsal structures.

Though WS and PB embryos were identical with respect to the absence of the dorsal and neural marker genes, they showed differences in *brachyury* expression. WS embryos showed *brachyury* expression in the constricted waist region (Fig. 5G), while PB embryos did not show *brachyury* expression at all (Fig. 5H).

NL embryos obtained by a slight deletion from the vegetal region showed circular expression of *goosecoid* (Fig. 5B) in contrast to the normal crescent-like expression (Fig. 5A). *brachyury* expression was also seen in NL embryos (Fig. 5F).

UV Irradiation and Lithium Treatment Resulted in the Formation of the Same Type of Hyperdorsalized Embryo

We have shown previously (Fujisue *et al.*, 1991) that cortical rotation occurs in Japanese newt embryos as well as in *Xenopus* embryos (Vincent *et al.*, 1986) and that the direction of the rotation accurately predicts the direction of the dorsal axis. The deletion experiment in the present study resulted in complete loss of dorsal structures in the newt embryos, as is the case in *Xenopus* embryos. These results strongly suggest that axis specification in newt embryos occurs by essentially the same mechanism as in *Xenopus*, namely, vegetally localized determinants move from the vegetal pole to a subequatorial region where they are incorporated into marginal cells to form the future organizing center (Sakai, 1996).

In *Xenopus*, UV irradiation is known to ventralize the resulting embryos (Malacinski *et al.*, 1977). This ventralization is accompanied by the loss of cortical rotation (Vincent and Gerhart, 1987b) so it follows that ventralization may be caused by the immobilization of the vegetally localized dorsal determinants (Sakai, 1996). In Japanese newt eggs as well, cortical rotation was observed to be entirely inhibited by UV irradiation (14 of 16 embryos did not show movement of the dye spots while the other 2 embryos showed cortical rotation; data not shown). Therefore, we expected that UV irradiation would have the same ventralizing effect on the newt embryos.

Surprisingly, most of the UV-irradiated Japanese newt embryos (12 of 14) showed a proboscis in the posterior region (Figs. 2C and 6A) which was reminiscent of those observed in LiCl-treated (hyperdorsalized) *Xenopus* embryos. We defined these embryos as HD embryos. At the time of gastrulation, the blastopore formed as an irregular ring (Fig. 2A), contrary to the normal I-shaped blastopore (Fig. 2P). At midgastrula, a pinhole-like blastopore was seen (Fig. 2B) while the ventral region of the normal embryos did not gastrulate at all during this stage (Fig. 2Q). During neurula to tailbud stages of the control embryos, HD embryos formed a proboscis in the posterior region (Figs. 2C and 6A). At this stage, a neural-fold-like structure (arrow-

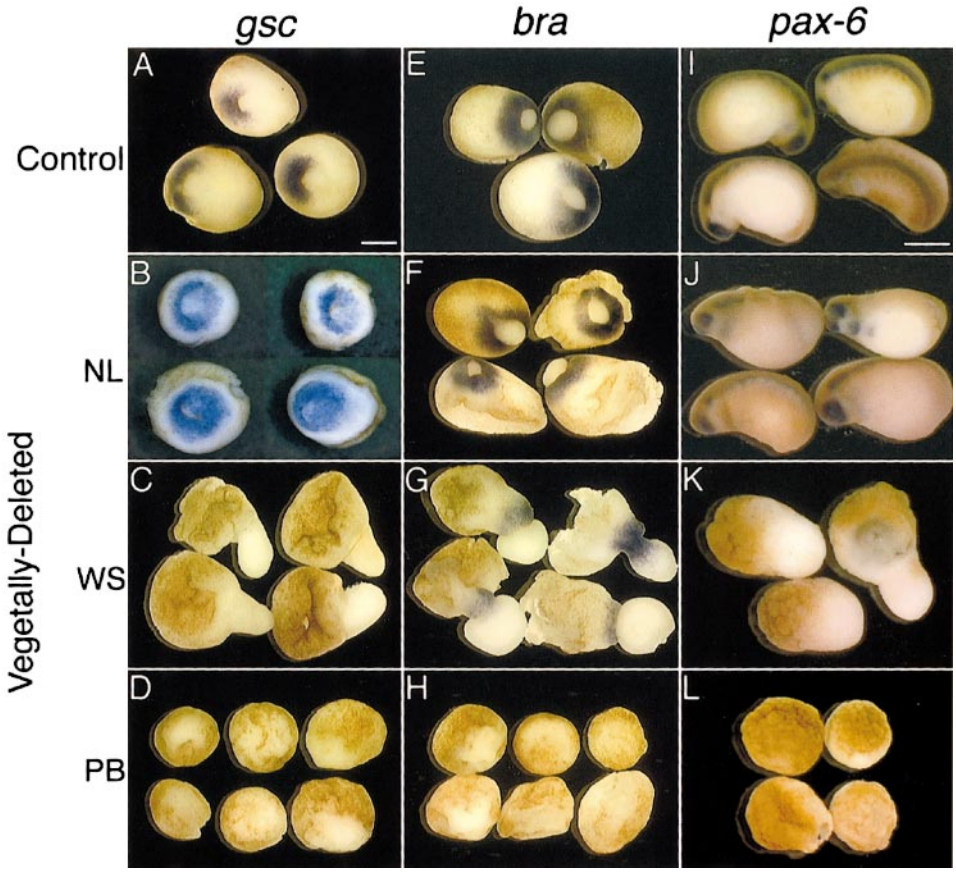


FIG. 5. Whole-mount *in situ* hybridization of the vegetally deleted embryos. (A–D) *goosecoid* expression at stage 12b. (E–H) *brachyury* expression at stage 13. (I–L) *pax-6* expression at stage 25. Top row: control embryos. Second row: NL embryos obtained by vegetal deletion. Third row: WS embryos. Bottom row: PB embryos. Neither WS (C) nor PB (D) embryos obtained by vegetal deletion showed expression of *goosecoid*, whereas NL embryos at the same stage (B) displayed a circular expression of *goosecoid*. Unexpectedly, *brachyury* expression in the WS embryos was observed in the constricted region (G). *Pax6* expression was seen in the eye rudiment and neural tube (I, J), while WS (K) and PB (L) embryos showed no expression of *pax6*. Bar in A for A–H and bar in I for I–L, 1 mm.

heads in Fig. 2C) appeared in the anterior ball region near the ball–proboscis boundary. In later development, the embryos usually degenerated from the proboscis region. Several examples of UV-irradiated embryos at stage 25 are shown in Fig. 6A. Although UV irradiation was usually carried out at a time of around 0.4, irradiation at later stages was also performed. Of 8 embryos irradiated at times later than 0.7, all developed normally (data not shown).

Although the external morphology of the UV-irradiated newt embryos suggests that these embryos are of a dorsalized type, histological examination did not confirm this conclusion. We found no clear differentiation of the notochord, somites, or neural tube (Fig. 2S) which were found in the NL embryos at the same stage (Fig. 2T). Therefore we investigated dorsal-specific gene expression. *goosecoid* mRNA was detected in the entire marginal zone of HD embryos at stage 12b (Fig. 6C), indicating that the dorsal marginal zone formed as a ring in UV-irradiated newt embryos. *brachyury* expression was observed in the marginal region of the UV-irradiated embryos at stage 13 (data not shown). *pax-6* expression was observed in the neural-fold-like region but not in the proboscis region of the stage 25 embryos, showing that the former is likely to be a neural structure.

Further, we examined the effect of LiCl treatment, which is known to hyperdorsalize *Xenopus* embryos, on newt embryos. As in *Xenopus*, Japanese newt embryos treated with LiCl exhibited a proboscis (17 of 20) similar to that observed in the UV-irradiated Japanese newt embryos (HD; Figs. 2F and 6B). Additionally, similar to our results with HD embryos formed through UV irradiation, we did not find any evidence of apparently differentiated notochordal tissues in the LiCl-treated embryos. These embryos expressed *goosecoid* (Fig. 6C), *brachyury* (Fig. 6D), and *pax-6* (Fig. 6F).

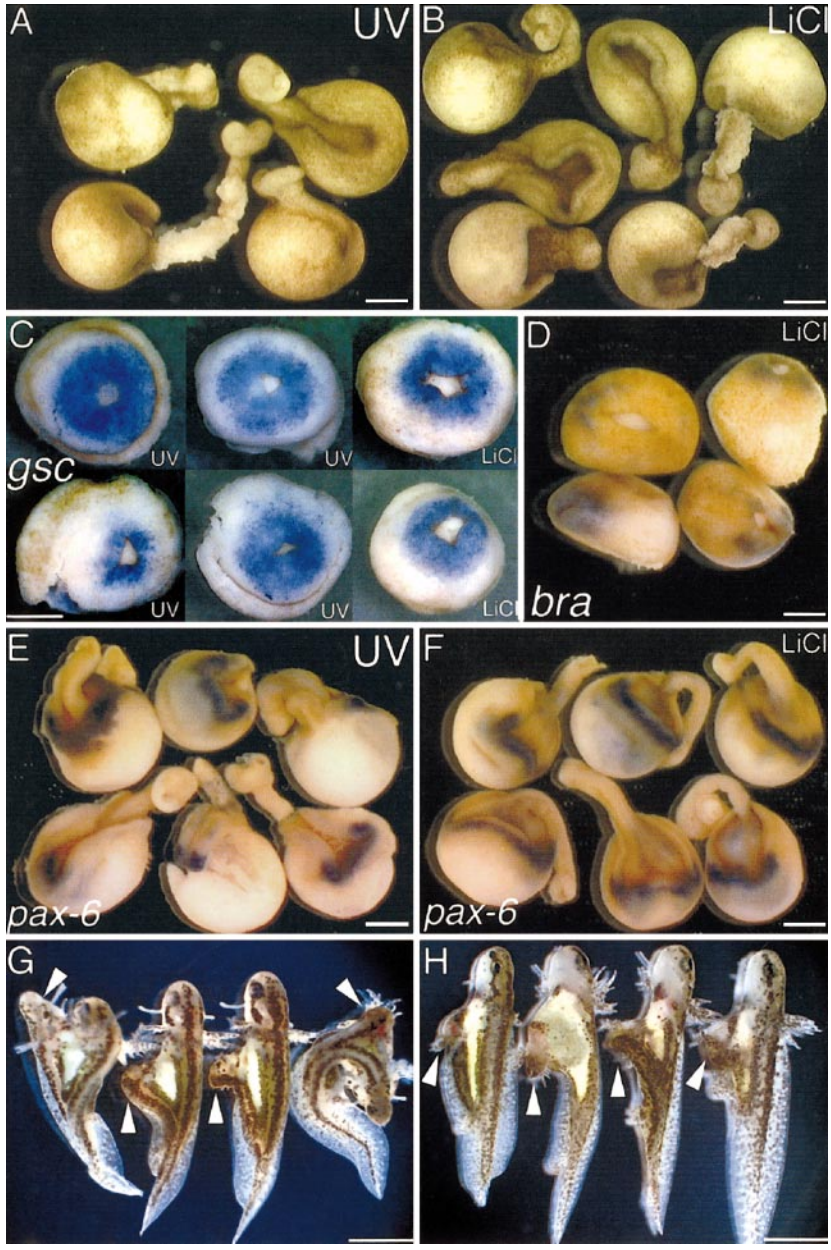


FIG. 6. UV irradiation and LiCl treatment hyperdorsalized newt embryos. (A) UV-irradiated embryos at the tailbud stage. (B) LiCl-treated embryos at the same stage. (C–F) Whole-mount *in situ* hybridization of UV-irradiated and LiCl-treated embryos. (C) *goosecoid* expression at stage 12b. UV, UV-irradiated embryos. LiCl, LiCl-treated embryos. *goosecoid* expression was observed around the circular blastopore. (D) *brachyury* expression in the LiCl-treated embryos at stage 13. (E, F) *pax-6* expression at stage 25. *pax-6* was expressed in the neural-fold-like region but not in the proboscis. (E) UV-irradiated embryos. (F) LiCl-treated embryos. (G) A series of organizer transplantation from a single UV-irradiated embryo. In this case, all four transplanted embryos formed a secondary axis (white arrowheads). (H) Organizer transplantation from a single LiCl-treated embryo. All four formed a secondary axis (white arrowheads). Note that in (H) and (G) all secondary embryos had gills. Bars in A–F, 1 mm. Bars in G and H, 2 mm.

Transplantation Using Entire Marginal Zone of the UV-Irradiated and LiCl-Treated Embryos

To examine whether the entire marginal zone of the UV-irradiated and LiCl treated embryos has organizer activity, marginal zone transplantations were performed on early gastrula (stage 12a) embryos. Transplantations were done in 100% MS with gentamycin (50 mg/L) on a bed of 2% agar. UV-irradiated and LiCl-treated donor embryos were labeled by 2% fluoresceinated-lysine dextran amine

(FDA; 10 nl per egg). Four donor tissues, 90° arc each, were excised from the marginal zone of a single donor embryo using a tungsten needle. The final size of the explants was about 1.0 mm width and 0.5 mm height. A cavity with dimensions similar to those of the donor grafts was made in the ventral marginal zone of the normal recipient embryos using a tungsten needle. Each graft was inserted into the cavity immediately after the explantation, and then the transplanted embryos were placed in round wells in the 2% agar bed. Positive and negative control experiments were done by transplanting normal dorsal marginal zone grafts and normal ventral marginal zone grafts, respectively. After a 2- to 5-h healing period, the transplanted embryos were placed in 10% MS with gentamycin (50 mg/L) and cultured for 2 weeks at 20°C.

We transplanted 76 grafts from 19 UV-irradiated donor embryos into the ventral marginal zone of the same stage recipients. Of these, 72 grafts induced secondary axes. A series of experimental embryos from a single UV-irradiated embryo is shown in Fig. 6G. In lithium-treated embryos, secondary axes were induced in 31 of 32 cases (see a sample in Fig. 6H). Histological analysis revealed that the progeny of donor grafts labeled with FDA were found in the dorsal structures such as notochord and neural tube in the induced secondary axes in both experiments (data not shown). In positive control experiments using the normal dorsal marginal zone, the secondary axes were induced in all cases ($n = 15$), while in negative control experiments using normal ventral marginal zone, no secondary axis was induced at all ($n = 12$). Thus the transplantation results definitely show that the entire marginal zone of UV-irradiated and lithium-treated embryos has organizer activity.

LiCl Hyperdorsalized WS but Not PB Embryos

Wasp-shaped embryos differed from permanent-blastula-type embryos at both a morphological and a molecular level. The WS embryos underwent self-constriction, and *brachyury* was expressed in the constricted region, whereas PB embryos did not show any sign of morphological movement and showed no *brachyury* expression. Therefore, it appears that WS but not PB embryos have a marginal zone. The observation that the WS embryos more frequently developed when smaller volumes were deleted from the vegetal pole (Fig. 4) also supports the notion that the WS embryos have a marginal zone while the PB embryos do not.

In *Xenopus*, DAI-0 embryos are hyperdorsalized by LiCl treatment (Sakai, 1996), but nongastrulating embryos are not (Sakai, unpublished observation). We therefore examined whether WS and PB newt embryos could be dorsalized by LiCl treatment. It is important to note that WS and PB embryos could not be distinguished at the time of treatment (32-cell stage), as described above. Accordingly, we lithium-treated vegetally deleted embryos with varying deleted volumes without knowing what types of embryo would emerge if lithium treatment were not carried out.

Vegetally deleted LiCl-treated embryos developed into two distinct forms: HD and PB embryos (Fig. 7A). NL and WS embryos were not observed following LiCl treatment. Since vegetal deletion alone can produce NL, WS, and PB embryos, we examined the fates of embryos in varying deletion ranges in order to determine which type(s) of embryo responded to LiCl treatment by forming HD and PB embryos. As shown in Fig. 4, deletion of more than 48% of the surface area inevitably resulted in formation of PB embryos. LiCl treatment of embryos in this deletion range also resulted in PB embryos without exception (Fig. 7B), suggesting that the fate of PB embryos was not altered by the LiCl treatment. In the 19–37% deletion range in the absence of LiCl, NL and WS embryos resulted (see Fig. 4). Figure 7B shows that embryos in this deletion range, when treated with LiCl, developed solely into HD. These results indicate that, between 19 and 37% deletions, HD embryos obtained by LiCl treatment would have developed into NL or WS embryos in the absence of LiCl. Since no WS or NL embryos were observed, it appears that all WS and NL embryos developed into HD embryos in response to LiCl treatment.

goosecoid was expressed throughout the marginal zone of LiCl-treated HD embryos, but was not expressed at all in LiCl-treated PB embryos (Fig. 7C). *brachyury* expression was observed in the HD but not in the PB embryos (Fig. 7D). As in the LiCl-treated whole embryos, the vegetally deleted LiCl-treated HD embryos expressed *pax-6*, while on the other hand, *pax-6* was not observed in the PB embryos (Fig. 7E).

DISCUSSION

The African clawed toad *X. laevis* is now a standard model for the study of early vertebrate development. There are obvious advantages in standardization, particularly the rapid transfer of results between laboratories, but there are also problems, principally in that concentration on a single species can lead to unwarranted generalizations (Slack, 1991). Observations on *Xenopus* embryos may not be directly applicable to other amphibians such as the Japanese newt, *C. pyrrhogaster*.

We have therefore made a comprehensive study of the early steps of axis specification in Japanese newt embryos, using methods previously applied to *Xenopus* embryos. The results show that there are several features common to early development in *Xenopus* and *Cynops*, but there are also some important properties which are specific to newt embryos.

The Dorsoanterior Classification for Newt Embryos

We classified four types of embryos with distinct dorsal axial development. Hyperdorsalized embryos were obtained when the embryos were UV irradiated or treated with LiCl.

We assumed tentatively that these embryos were "hyperdorsalized" because of their external morphology: they formed a characteristic protrusion in the posterior (original vegetal) region which resembled the proboscis formed in hyperdorsalized (LiCl-treated) *Xenopus* embryos (Kao *et al.*, 1986). Further, HD embryos formed neural-fold-like structures at the base of the protrusion.

Molecular studies confirmed that HD is likely to be a hyperdorsalized form. At stage 12b, *goosecoid*, a dorsal marker gene (Sone *et al.*, 1997), was expressed throughout the entire marginal region, and at the tailbud stage *pax-6* (a neural marker) was expressed in the neural-fold-like structure.

Normal-looking embryos were normal with respect to both the morphological and the molecular characteristics examined.

Wasp-shaped embryo is a new form which has not been reported previously. This type of embryos shares some properties with DAI-0 embryos of *X. laevis*. Both WS newt embryos and DAI-0 *Xenopus* embryos develop from vegetally deleted embryos and lack dorsal structures. Expression of *brachyury* and dorsalization by LiCl are also common features in these embryos.

Permanent-blastula-type embryos did not express *brachyury* and were not dorsalized by LiCl, showing that these embryos were different from WS embryos. We think that PB embryos are homologous to nongastrulating *Xenopus* embryos, which develop from deletion of more than 60% vegetal surface area (Sakai, 1996).

General "Determinant Model" of Axis Formation in Amphibian Embryos: Properties Common to both Xenopus and Cynops

We have previously shown that deletion of vegetal cytoplasm resulted in the complete loss of dorsal structures in *Xenopus* embryos (Sakai, 1996). We proposed a model for the formation of the dorsal axis in *Xenopus* embryos in which components of two specific cytoplasmic regions are necessary for formation of the organizer: vegetal cytoplasm is required for dorsalization and marginal zone cytoplasm is necessary for marginal endomesodermal differentiation. The presence of dorsal determinants (DDs) in the vegetal cytoplasm was hypothesized because deletion of the vegetal region resulted in the loss of dorsal structures. The importance of marginal zone cytoplasm was indicated by the fact that gastrulation in *Xenopus* was not inhibited by deletion of moderate proportions (20–40%) of the vegetal cytoplasm. It has been recently proposed that β-catenin (Larabell *et al.*, 1997) and Dishevelled (Miller *et al.*, 1999) are involved in the dorsal determinant system.

The present results in Japanese newt embryos generally support and strengthen our determinant hypothesis. Loss of the dorsal axis resulting from vegetal deletion suggests that the determinants of dorsal axis formation in the Japanese newt are confined to the vegetal region of the newly fertilized egg. Deletion of the vegetal region in two species of fish embryos is also reported to inhibit dorsal axis formation (Mizuno *et al.*, 1997b, 1999). The hyperdorsalization by UV irradiation in the Japanese newt seems, at first sight, to contradict the determinant hypothesis. However, if we assume that the dorsal determinants and the marginal cytoplasm overlap in the marginal region, the fact that UV irradiation caused hyperdorsalization could be reasonably explained. This point will be discussed later.

Therefore, the localization of dorsal determinants in the vegetal pole may be a feature common to many species within the Anamnia. Further, the present study provides new information regarding the marginal cytoplasm. Wasp-shaped embryos which do not form dorsal structures can express the marginal zone-specific gene *brachyury*. *brachyury* was expressed in the constricted region of the WS embryos but not in the PB embryos. It therefore appears that WS embryos contain marginal cytoplasm while PB embryos do not. The fact that PB embryos were obtained more frequently when the deleted volume was greater supports this notion.

It should be noted that *brachyury* expression did not extend to the vegetal pole region of WS embryos. It was always expressed in the constricted region, which overlaid the yolky vegetal cells. In *Xenopus, brachyury* (*Xbra*) is thought to be expressed through inductive processes (Lemaire and Gurdon, 1994). The *Xbra* expression region in *Xenopus* overlays the *Xwnt-8* expression region (Lemaire and Gurdon, 1994). In Japanese newts, it is possible that *brachyury* expression is similarly induced by vegetal cells.

Although ventralized Japanese newt embryos (WS embryo) are unique in several respects (see below), they could be hyperdorsalized by LiCl, as can *Xenopus* ventralized (DAI-0) embryos, which suggests a degree of similarity between ventralized *Xenopus* and newt embryos.

The observations of self-constriction, *brachyury* expression, and hyperdorsalization by lithium in the WS but not in the PB embryos strongly support the presence of specific marginal cytoplasm in the WS embryos.

Differences between Xenopus and Newt Development

Vegetal determinants may be spread more widely in newt embryos. Compared to *Xenopus* embryos, a greater volume of vegetal cytoplasm had to be deleted from newt embryos to induce the loss of dorsal structures. Thus, it seems likely that the dorsal determinants are spread wider in newt than in *Xenopus* embryos. This wide distribution of dorsal determinants may be the basis of hyperdorsalization by UV irradiation, as discussed below. Normal-looking embryos that developed from the vegetally deleted embryos usually showed circular expression of *goosecoid*. This may be due to the surgical process which partially inhibits the cortical rotation; this may cause semicircular distribution of the dorsal determinants in the marginal zone which in turn direct the expression of organizer-specific genes such as *goosecoid*.

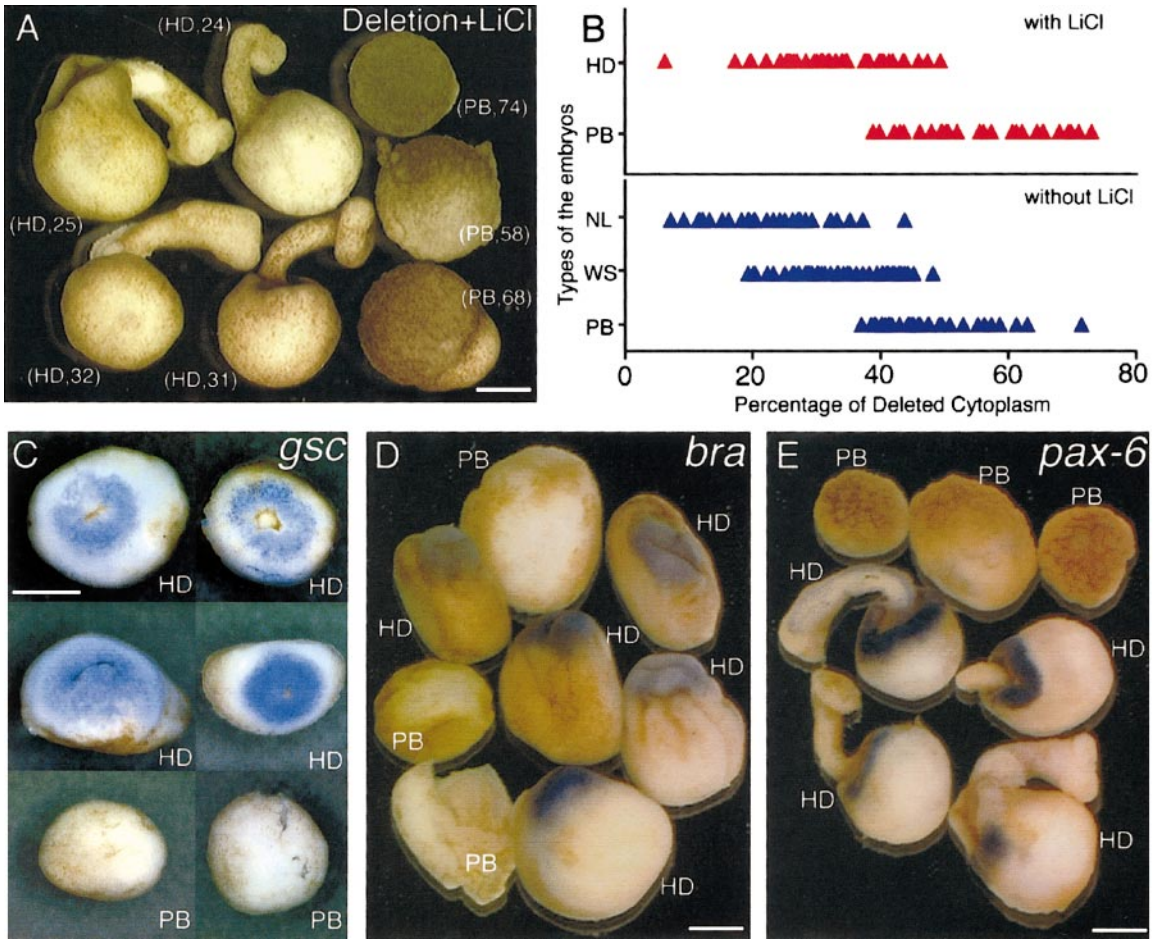


FIG. 7. LiCl treatment hyperdorsalized NL and WS but not PB embryos. (A) LiCl-treated embryos from which vegetal cytoplasm had already been deleted. The abbreviations for the classes of morphology and deleted volumes are shown in parentheses as in Fig. 3. The resulting embryos shown here, without exception, developed into HD and PB embryos. NL and WS embryos were not observed. (B) Morphology of LiCl-treated embryos plotted against the deleted volume. Top: Vegetally deleted LiCl-treated embryos. Bottom: Vegetally deleted embryos that were not treated with LiCl. This graph shows the same data as in Fig. 4. (C–E) Whole-mount *in situ* hybridization of HD and PB embryos obtained by vegetal deletion followed by LiCl treatment. (C) Expression of *goosecoid* in HD and PB embryos at stage 12b. (D) *brachyury* expression at stage 13. (E) *pax-6* expression at stage 25. Note that *goosecoid*, *brachyury*, and *pax-6* were expressed only in HD embryos. In C–E, the abbreviations for the classes of morphology are shown for each embryo. All bars, 1 mm.

Self-constriction movement is a specific property of the ventral marginal zone in Japanese newt embryos. In the present study, we have shown, for the first time, self-constriction movement in newt embryos. To our knowledge, this type of movement has not been reported in newt or other amphibian species. In *Xenopus*, vegetally deleted embryos which lack dorsal structures gastrulate when the ventral marginal zone of the normal control embryos begins to gastrulate (ventral gastrulation). In Japanese newt, self-constriction movement seemed to replace the ventral gastrulation movement seen in *Xenopus* embryos.

The expression of *brachyury* in the constricted region of WS embryos suggests that *brachyury* expression may be involved in self-constriction movement. In *Xenopus*, *Xbra* is thought to be a mesodermal marker and concerned with gastrulation movement (Yamada, 1994). Ventralized *Xenopus* embryos express *Xbra* (Fujii and Sakai, unpublished observation) and these embryos form ventral mesoderm derivatives such as mesenchyme and blood (Sakai, 1996; Scharf and Gerhart, 1980). Although WS embryos expressed *brachyury*, which shows mesoderm formation, these embryos neither gastrulated nor formed mesodermal derivative tissues in later stages. Therefore, gastrulation and the formation of mesodermal derivatives in Japanese newt embryos appear to require additional factors other than *brachyury*.

WS embryos at the tailbud stage, at first sight, seem to resemble to exogastrula embryos (Holtfreter, 1933) but

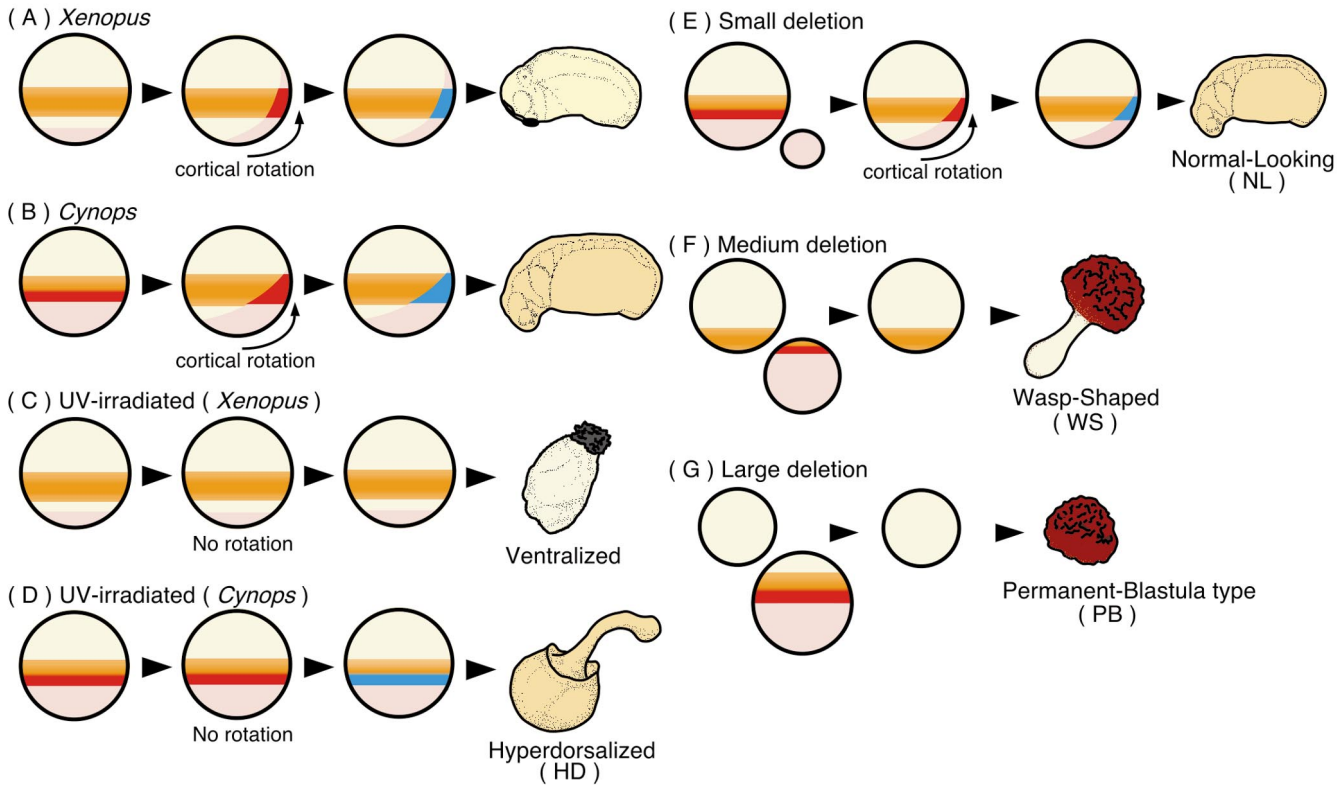


FIG. 8. Proposed model of the early steps of axis formation of Japanese newt embryos in comparison to the *Xenopus* model. (A) Model for normal axis formation in *Xenopus*. (B) Proposed model for normal axis formation in Japanese newt. In both animals, there are two specific regions important for later development: the vegetal pole region, which carries dorsal determinants (DDs; pink) and the subequatorial circular marginal region (orange), which requires the dorsal signal to form the dorsal marginal zone (Spemann organizer; blue). The difference between the two species is that, at the onset of development, the vegetal region and the marginal region in newt embryos share the circular subequatorial area (red), whereas in *Xenopus* the two regions are separated. Overlapping (mixing) of the two specific cytoplasms (red) results in Spemann organizer formation at a later stage. In *Xenopus*, cortical rotation translocates the vegetal DDs to a sector of the marginal region where the organizer later forms (A). On the contrary, in newts, the main role of cortical rotation is to eliminate the DDs from the future ventral region (B). In both *Xenopus* and the Japanese newt, UV irradiation fixes the original situation of the two specific cytoplasms; however, the outcome is quite different (C, D). In *Xenopus* (C), the DDs do not overlap the marginal region in the absence of cortical rotation, hence the Spemann organizer does not form. In newt (D), the overlapping region is fixed so that the organizer forms in the circular subequatorial region. The deletion of the newt vegetal cytoplasm (E–G) in principal results in the same outcome as in *Xenopus;* however, moderate deletion of 19 to 48% results in newt-specific phenotype (F). This is because the marginal region of newt does not have the ability to gastrulate. Gastrulation is a dorsal-specific property in newt embryos. The marginal region of newt with no DDs instead can constrict and express *brachyury*.

these two phenotypes probably reflect different abnormalities in gastrulation. Incomplete blastopore closure in the WS newt embryos arises from a lack of convergent extension of the axial mesoderm, while exogastrulation occurs when mesodermal convergent extension is misdirected, and the axial mesoderm extends outward from the embryo as a result. In fact, our WS embryos never formed dorsal mesodermal tissues, while exogastrula embryos form the notochord, somite, and head mesoderm. Dorsal marker genes are expressed in the *Xenopus* exogastrula (Kintner and Melton, 1987; Taira *et al.*, 1994) but not in WS newt embryos.

Gastrulation is a specific property of the dorsal marginal zone in newt embryos. Vegetally deleted ventralized *Xenopus* embryos form a blastopore, indicating that gastrulation in *Xenopus* is independent of DDs. In the present study, gastrulation did not occur in Japanese newt embryos when the dorsal axis did not form. Vegetally deleted newt embryos could gastrulate (when the deleted portion was small), but these embryos without exception formed dorsal structures. Further, WS embryos were dorsalized by LiCl treatment and then developed into hyperdorsalized embryos. In these embryos as well, gastrulation was always accompanied by the formation of dorsal axial structures.

Thus in Japanese newt embryos, gastrulation was always accompanied by dorsal axis formation. In other words, we found no "ventral gastrulation" in newt embryos. This suggests that gastrulation and mesoderm formation in the Japanese newt embryo require not only a marginal factor but also a dorsalizing factor.

UV irradiation dorsalized newt embryos. One of the most striking results of the present study is that UV irradiation hyperdorsalized the resulting embryos. In addition to a characteristic proboscis reminiscent of *Xenopus* hyperdorsalized embryos, UV-irradiated HD embryos expressed *goosecoid* mRNA throughout the marginal zone of stage 12b embryos and expressed *pax-6* in the neural-fold-like region but not in the proboscis region of the stage 25 embryos (Fig. 8C). Transplantation experiments showed that the entire marginal zone of the UV-irradiated Japanese newt embryos has specific organizer activity. These results were unexpected because UV irradiation of *Xenopus* embryos is known to exert the opposite (ventralizing) effect (Malacinski *et al.,* 1977; Scharf and Gerhart, 1980, 1983), which is thought to be the result of interference in cortical rotation (Vincent and Gerhart, 1987b). In addition, in the European newt *Pleurodeles waltl,* UV irradiation ventralized the embryo (Shi *et al.,* 1989). Lithium treatment results in hyperdorsalized embryos in *Xenopus* (Scharf *et al.,* 1989) and *Pleurodeles* (Shi *et al.,* 1990), just as in the Japanese newt in the present study. Given that the fundamental mechanism for axis formation in the Japanese newt embryos appears to be identical to that in *Xenopus* and in the European newt, we thought that DDs in UV-irradiated Japanese newt embryos would be fixed in the vegetal region. Indeed, cortical rotation was not observed in UV-irradiated newt embryos.

One possible explanation for these observations is as follows. In *Xenopus,* the region containing DDs and the marginal zone do not overlap in the early postfertilization stages of development. UV irradiation fixes this initial situation so that the DDs never reach the marginal zone. In the Japanese newt, the marginal zone most likely overlaps with the DD domain in the early postfertilization stages. Deletion experiments revealed that the DDs appear to be more widespread in the Japanese newt than in *Xenopus,* supporting the idea of overlapping vegetal and subequatorial domains. The overlapping of the DD domain and the marginal zone is most probably fixed by UV irradiation, and the overlapping region may then develop into the organizer.

Proposed Model for Dorsal Axis Specification during the First Cell Cycle

Based on the present study, we propose a model for the specification of the dorsal axis in *C. pyrrhogaster* (Fig. 8) by comparison with our previous model for *Xenopus* embryos in which DDs localized in the vegetal region seem to be distributed more widely in newt than in *Xenopus* embryos (Fig. 8B). In this model, overlapping (mixing) of the two specific cytoplasms results in Spemann organizer formation in a cell-autonomous manner. We think that during the cleavage period, intracellular processes such as cytoplasmic mixing play a crucial role since we have recently found that the dorsalizing induction from the dorsal vegetal cells of *X. laevis* occurs only after midblastula transition (Nagano *et al.,* 2000), which is inconsistent with the idea of cleavage-stage mesoderm induction. Although the present results provide no information on the timing of the inductive processes concerned, our results with *Xenopus* embryos suggest a possibility that the dorsal axis formation in the Japanese newt also proceeds via an intracellular mode of development.

In *Xenopus,* the dorsalizing effects of lithium are presumably due to the stabilization and accumulation of β-catenin, which result from the inhibition of glycogen synthase kinase-3 (GSK-3) (Klein and Melton, 1996; Larabell *et al.,* 1997). Although we did not examine these molecules in our Japanese newt embryos, the similarity of the lithium effects in both *Xenopus* and Japanese newt embryos suggests that Japanese newt embryos use an identical molecular system for the dorsalization of the marginal zone. Presumably, our WS embryos can be hyperdorsalized by lithium because β-catenin, GSK-3, and other molecules involved are still present in amounts sufficient for a response to lithium. *goosecoid* expression in the lithium-treated embryos was always observed in a circular region just above the blastopore, suggesting that the lithium-responding system is active only in this region. Our PB embryos failed to respond to lithium most likely because these embryos lack one or more of the molecules in the β-catenin-induced dorsalizing pathway.

Although we propose here that the entire marginal region has specific cell-autonomous factors, it is also possible that cells of the vegetal region might induce certain processes in the marginal zone. *brachyury* expression in the WS embryo was always observed in the constriction region overlaying a yolky endodermal region. In what may be an analogous situation, a vegetally localized maternal gene transcript is thought to be required for mesoderm formation in *Xenopus* (Zhang *et al.,* 1998).

The present study revealed that deletion of vegetal cytoplasm resulted in two types of axis-deficient embryo in Japanese newts. These embryos may provide a useful model system for elucidating inductive interactions within the developing embryo. Although neither type of embryo formed dorsal structures, self-constriction movement and *brachyury* expression were observed only in WS embryos. In addition, LiCl treatment affected only WS embryos. Other treatments such as organizer transplantation may also have different effects on these two types of embryos.

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